Labjournal Landmine Detection

01.07.2014
Grow on shakeflasks the strains received from Belkin lab (J::GFP, F::GFP, J::lux, F::lux, FB2::lux, FB2A1::lux) and the host E. coli strain C43(DE3). F is the promoter from the yqjF gene from Escherichia coli. J is the promoter from the ybiJ gene from Escherichia coli. FB2 and FB2A1 are improved versions of the yqjF promoter generated via mutagenesis.

02.07.2014
Miniprep Belkin samples (J::GFP, F::GFP, J::lux, F::lux, FB2::lux, FB2A1::lux)
Glycerol stock of Belkin samples (same as above) and the host C43(DE3)

16.07.2014
Miniprep + Glycerol Stocks of mKate2

Measured the concentration of the isolated DNA with nanodrop:

- mKate2 39.2 ng/ul

22.07.2014

PCR ybiJ from Belkin ybiJ:lux	Amnt.	(ul)
Template: ybiJ:lux [233]	75ng total	0.35
FW ybiJ (5uM)	5pm	2.5
RV ybiJ (5uM)	5pm	2.5
dNTP (10mM)		1.5
pFx		0.2
Buffer 10x		5
MgSO4 (50mM)		1
Enhancer		5
MQ		32
		50

PCR ybiJ from Belkin ybiJ:GFP	Amnt.	(ul)
Template: ybiJ:GFP [72]	50ng total	1
FW ybiJ (5uM)	5pm	2.5
RV ybiJI (5uM)	5pm	2.5

dNTP (10mM)		1.5
pFx		0.2
Buffer 10x		5
MgSO4 (50mM)		1
Enhancer		5
MQ		31.3
		50

Negative	Amnt.	(ul)
FW ybiJ (5uM)	5pm	2.5
RV ybiJ (5uM)	5pm	2.5
dNTP (10mM)		1.5
pFx		0.2
Buffer 10x		5
MgSO4 (50mM)		1
Enhancer		5
MQ		32.3
		50

PCR mKate2	Amnt.	(ul)
Template: mKate2 [39.2]	78.4 ng total	2
FW ybiJ (5uM)	10 pmol	2
RV ybiJI (5uM)	10 pmol	2
dNTP (10mM)		1.5
pFx		0.2
Buffer 10x		5
MgSO4 (50mM)		1

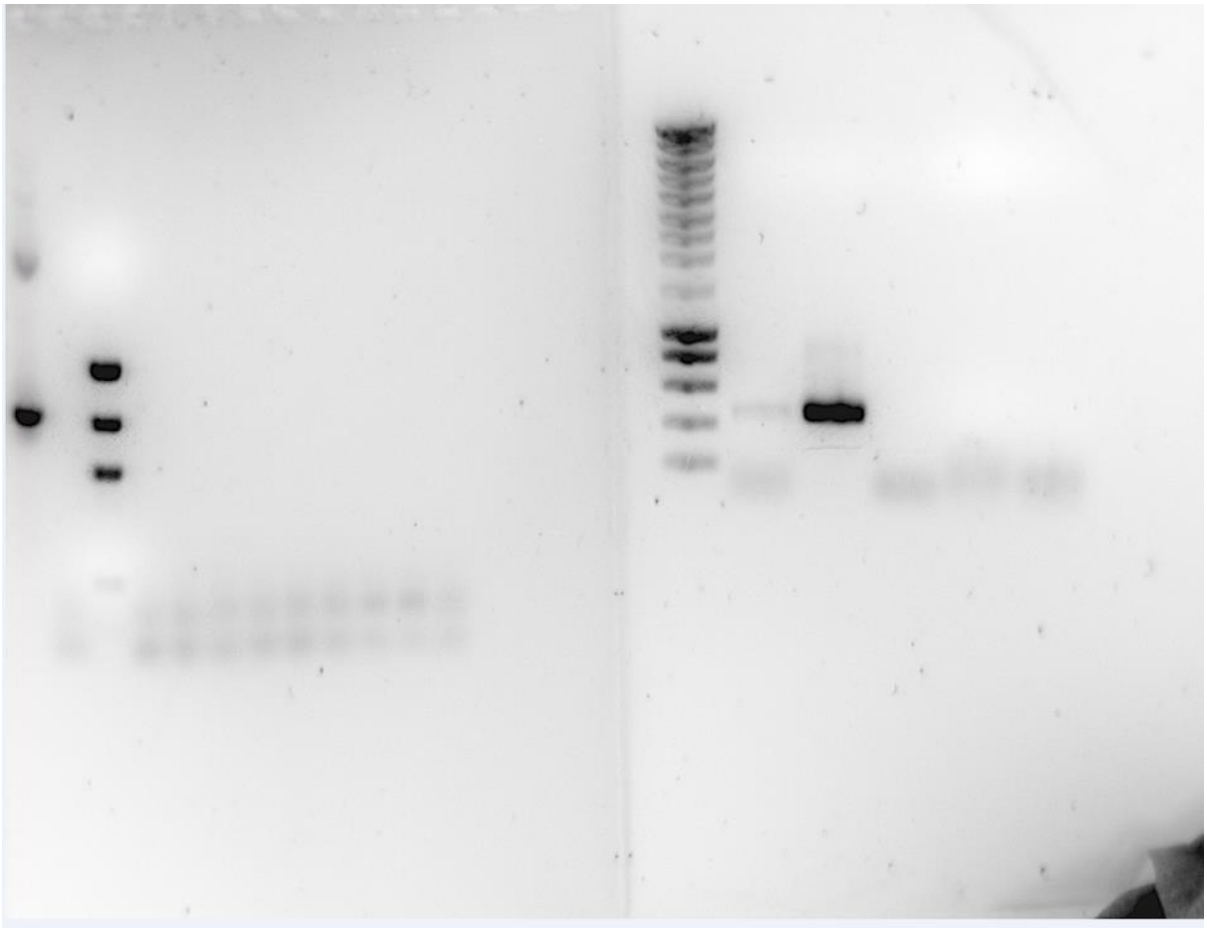
Enhancer		5
MiliQ		31.3
		50

Negative control of mKate2	Amnt.	(ul)
FW ybiJ (5uM)	10 pmol	2
RV ybiJ (5uM)	10 pmol	2
dNTP (10mM)		1.5
pFx		0.2
Buffer 10x		5
MgSO4 (50mM)		1
Enhancer		5
MQ		33.3
		50

PCR program	t	T (C)
1	5’	95
2	30”	95
3	30”	62
4 → step 2	1’	68
5	5’	72
6	etern.	4

Run a 1% Agarose gel with the PCRed samples. The lanes of the gel correspond to:

1. Smart ladder
2. PCR ybiJ from Belkin ybiJ:lux
3. PCR ybiJ from Belkin ybiJ:GFP
4. Negative
5. PCR mKate2
6. Negative control of mKate2



The ybiJ promoter from Belkin ybiJ:GFP was successfully amplified. mKate2 was not.

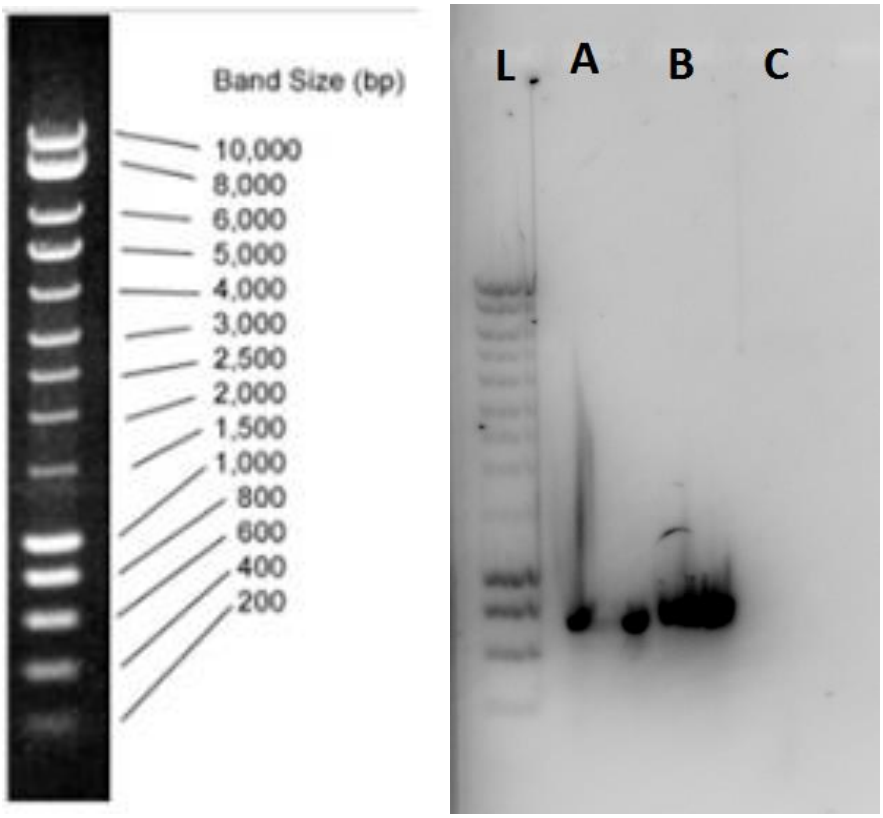
23.07.2014
Purified ybiJ PCR products from ybiJ:GFP and ybiJ:lux templates using QIAquick kit. 45 ul of PCR product were used for the purification. DNA concentration of the purified ybiJ: 66.2 ng/ul.

Repeat PCR for mKate2 with exactly the same elements as the mKate2 PCR on 22.07.2014, but using 0.35 ul of pfx polymerase instead of 0.2ul (therefore, using 31.2 ul of MiliQ instead of 31.3 ul). As a DNA template, this time mKate2 plasmids miniprepped on two different days were used: Red label and Blue label

24.7.2014

A 1% agarose gel was run. Lanes:

1. Smart ladder
2. PCRed mKate2 (red labelled)
3. PCRed mKate2 (blue labelled)
4. Negative control



Lane B (PCRed mKate2 (blue labelled)) is around the 800 bp band, which is the expected size of the amplified mKate2 (756 bp). The concentration of the purified mKate2 is 193 ng/ul.

31.07.2014
Anne

PCR of PyqjF
PCR mix 2x25 :

	ul
template	1
FW	3
RV	3
mgso4 50 mM	1
dNTP 10 mM	1,5
pfx buffer 10x	5
enhancer	5
pfx polymerase	0,4
MQ	30,1
total	50

Template: B2A1 [200ng/ ul] (Belkin plasmid with yqjF promoter)
FW: yqjF belkin forward[5uM]
RV: yqjF belkin reverse good RV [5uM]

PCR cycling conditions:

cycling conditions	minutes	°C	
1	3	94	
2	1	94	
3	1	54.9 and 57.8	
4	1	68	go to step 2, 29x
5	5	68	

-> 4 degrees

31.07.2014
Tomek
PCR for gibson assembly of mKATE, Vector (x2), ybjF promoter and yqiJ promoter.

PCR Scheme for mKATE-GB (1)

	ul
template	2,2
FW	1,5
RV	1,5
mgso4 50 mM	0,5
dNTP 10 mM	1,75
pfx buffer 10x	2,5
enhancer	2,5
pfx polymerase	0,4
MQ	13,35
total	25

PCR Scheme for ybjF-GB (2)

	ul
template	1
FW	1,5

RV	1,5
mgso4 50 mM	0,5
dNTP 10 mM	0,75
pfx buffer 10x	2,5
enhancer	2,5
pfx polymerase	0,4
MQ	14,45
total	50

PCR Scheme for ybjF-GB (3)

	ul
template	1,3
FW	1,5
RV	1,5
mgso4 50 mM	0,5
dNTP 10 mM	0,75
pfx buffer 10x	2,5
enhancer	2,5
pfx polymerase	0,4
MQ	14,25
total	25

PCR Scheme for ybjF-GB (4)

	ul
template	0,5
FW	1,5
RV	1,5
mgso4 50 mM	0,5
dNTP 10 mM	0,75

pfx buffer 10x	2,5
enhancer	2,5
pfx polymerase	0,4
MQ	14,95
total	25

PCR Scheme for ybjF-GB (5)

	ul
template	0,5
FW	1,5
RV	1,5
mgso4 50 mM	0,5
dNTP 10 mM	0,75
pfx buffer 10x	2,5
enhancer	2,5
pfx polymerase	0,4
MQ	14,95
total	50

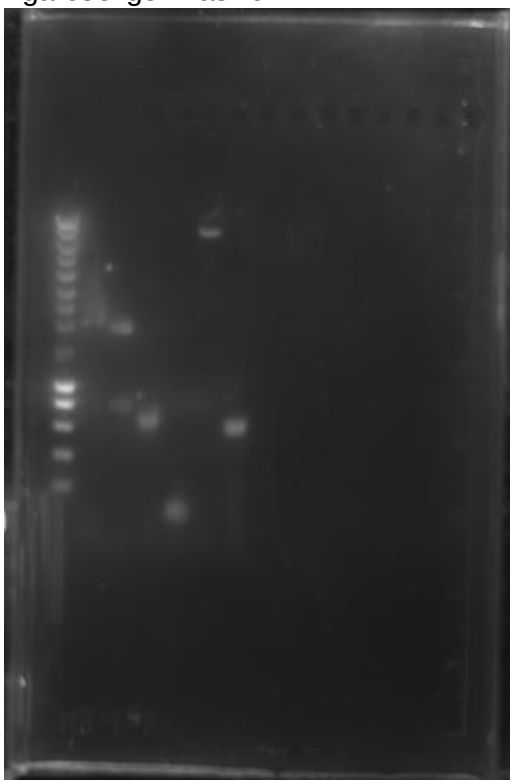
PCR cycling conditions:

cycling conditions	minutes	°C	
1	3	95	
2	1	95	
3	1	55.0 and 57.8	
4	1	68	go to step 2, 29x
5	5	68	

-> 4°C

01.08.2014
Tomek
Making mastermix for Gibson assembly.

Joan
For the construction of the [Land mine promoter + mKate2 + DT], the following 1% Agarose gel was run:



Lanes 1, 2 and 4 correspond to: Smart Ladder; plasmid containing the DT cut with EcoRI and XbaI; and mKAte2 cut with EcoRI and PstI respectively. Both lanes 2 and 4 seem to contain the desired part. The DT band, however, looks a bit smirred

04.08.2014
Janna, Joan, Mariëlle, Anne

Colony PCR of potential transformants with mKate + double terminator construct. Six colonies and one colony that appeared on the plate without insert were checked.

PCR mix:

	ul
FW mkate (5uM)	2.5
RV mkate (5uM)	2.5
template = single colony	/
Taq master mix 2x concentrated	12.5
Taq polymerase	0.2
MQ	7.3

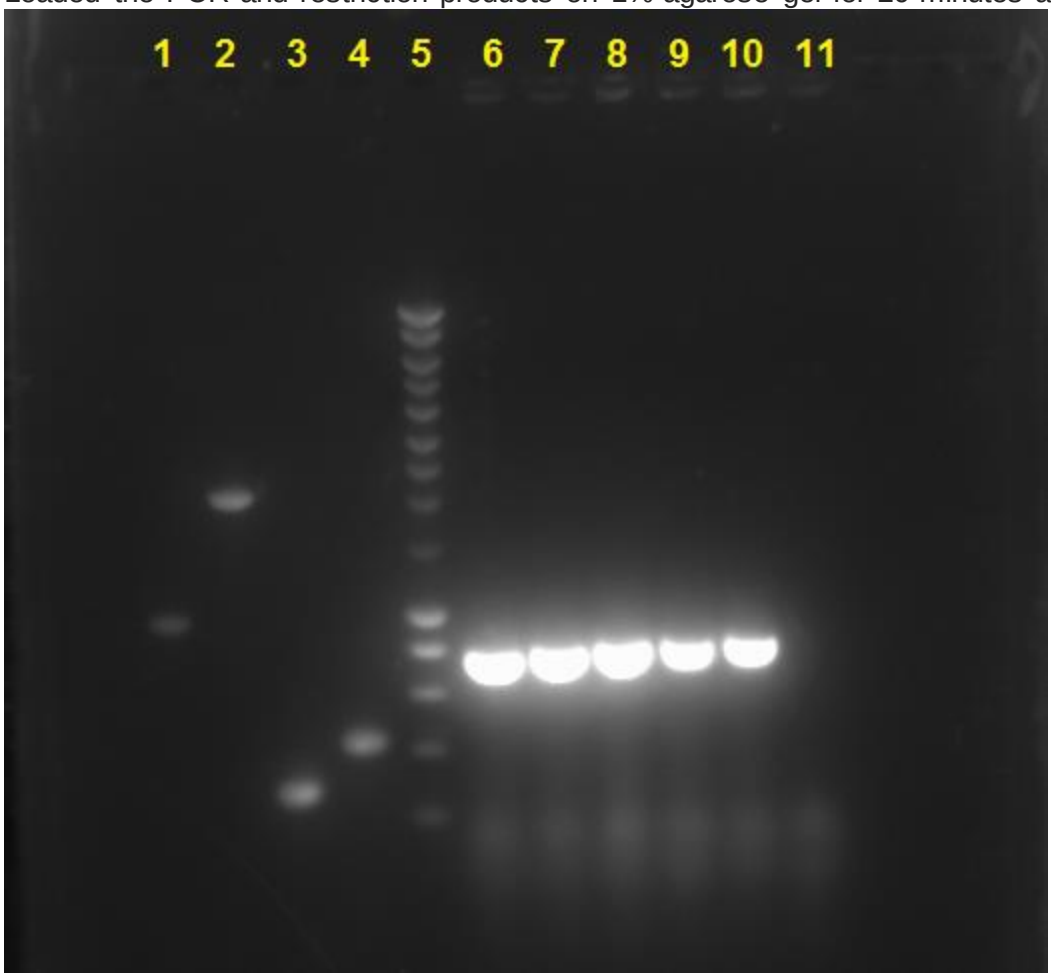
Cycle conditions

cycling conditions	minutes	°C	
1	5	94	
2	1	94	
3	1	55	
4	1	72	go to step 2, 29x
5	5	72	

→ 4 degrees

PCR result

Loaded the PCR and restriction products on 1% agarose gel for 20 minutes at 100 V.



PCR of different small samples and mKate.

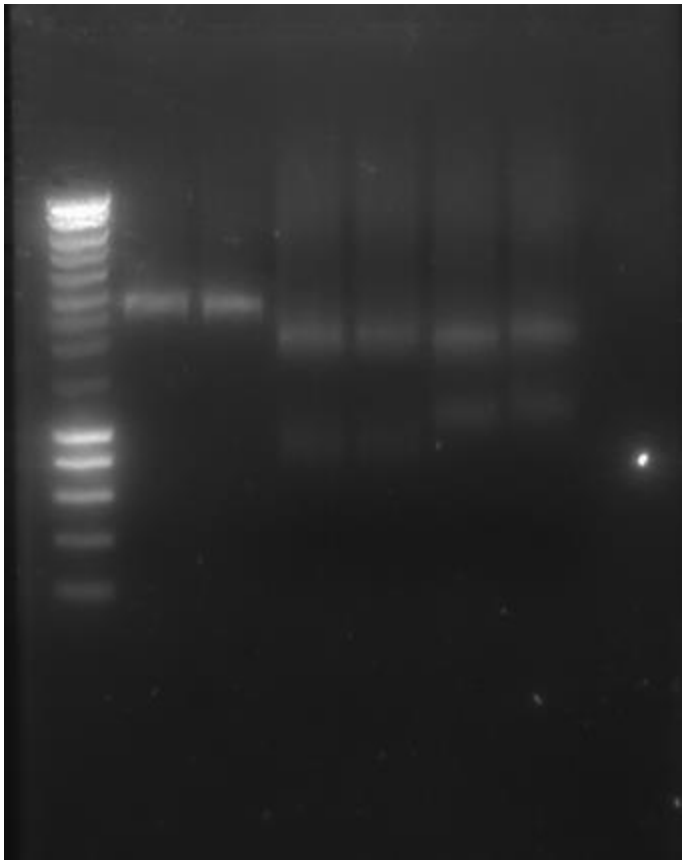
1. CsgBA)
2. Rhamnose promoter cut with S and P)
3. LD Promoter F cut with E and S (expected size ….)
4. LD Promoter J cut with E and S (expected size ….)
5. Marker
6. mKate first colony (15 ul with 5 ul of loading dye) (expected size ….)

7. mKate second colony (15 ul with 5 ul of loading dye)
8. mKate third colony (15 ul with 5 ul of loading dye)
9. mKate fourth colony (15 ul with 5 ul of loading dye)
10. mKate fifth colony (15 ul with 5 ul of loading dye)
11. mKate negative control (15 ul with 5 ul of loading dye)

05.08.2014
Anne and Janna:
Innoculated colonies from the plate with mKate-DT (1) in 3 ml liquid LB medium with chloramphenicol. We used numbers 1 and 2 from the plate with transformants from 04.08.2014. Put in the stove at 37 C for 5 hours. Made glycerolstock of culture, made minipreps of both cultures.

06.08.2014
Joan
Agarose Gel, from left to right:

- Smart Ladder
- LD1 from culture 1&2 restricted in both cases with EcoRI and XbaI, which would lead to an open plasmid (lanes 2-3)
- LD1 from culture 1&2 restricted in both cases with EcoRI and PstI (lanes 4-5), which leads to 1 band of the biobrick, and a 2nd band of the backbone
- Lanes 6 and 7 are from the CC module (CC52 cult 1 and 2 cut with EP)



Purify LD1 from lanes 2&3. DNA concentration:

- Culture 1: 20.2 ng/ul
- Culture 2: 139 ng/ul

07.08.2014
Anne and Janna
Colony PCR of potential transformants with LD promoter L or F and mKate

Per promoter two different cultures (based on mKate + DT construct) were tested: c1 and c2 and 6 colonies per culture were tested.

Master mix contained:

	ul	16x
TAQ MM 2x	12.5	200
FW [5uM]	2.5	40
RV [5uM]	2.5	40
MQ	7.5	120
total	25	400

Primers promoter J:

ybiJ FW
ybiJ RV

Primers promoter Q

yqjF FW
yqjF rev good

MM was aliquoted in 25 ul samples. As negative control a colony that appeared on the MQ plate (which means: insert=MQ) was used as template. As positive control the belkin plasmids with associated promoters were used.

Colonies were ented with a pipet tip, inoculated in 25 ul MM, and streaked on LB+ CAM.

Cycle conditions

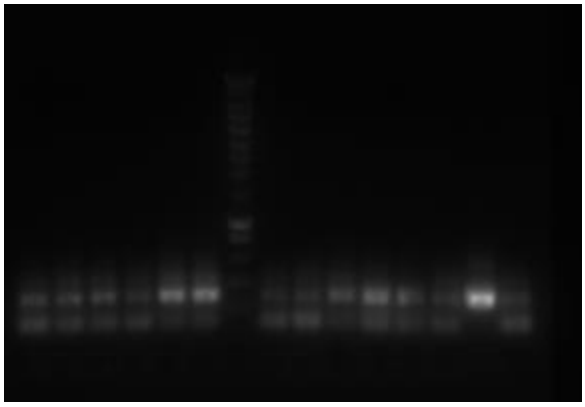
cycling conditions	minutes	°C	
1	5	94	
2	1	94	
3	1	56	

4	1	72	go to step 2, 29x
5	5	72	

→ 4 degrees

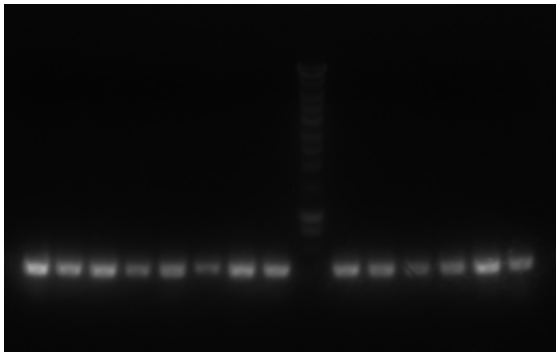
add 5 ul laoding dye to PCR mixtures
add 10 ul sample to a 1 % agerose gel, 100V 30 minutes

from left to right
1-6 potential p[F]-mkate (306bp)
Smart-ladder marker
7-12 potential p[F]-mkate (306bp)
+ control
- control



For all colonies the expected fragment is observed. In addition, a lower fragment is observed as well. This could be primer dimers. The negative control is not negative, this might be due to binding of the primers to genomic DNA of E.coli, since the promoter is present in WT E. coli as well. Therefore the PCR will be repeated using a mKate reverse primer instead of a reverse primer annealing to p[F]

From left to right
1-6 potential p[J]-mkate (459 bp)
+ control
- control
Smartladder
7-12 potential p[J]-mkate (459 bp)



For all colonies the expected fragment is observed. The negative control is not negative, this might be due to binding of the primers to genomic DNA of E.coli, since the promoter is present in WT E. coli as well. Therefore the PCR will be repeated using a mKate reverse primer instead of a reverse primer annealing to p[J]

08.08.2014
Anne and Janna

Colony PCR of promoter J and F
Made the following MasterEps:

MasterEp J

FW [J]	2.5 ul	x 16 =	40 ul
RV [mKate]	2.5 ul	x 16 =	40 ul
2 x MasterMix TAQ	12.5 ul	x 16 =	200 ul
MilliQ	7.5 ul	x 16 =	120 ul
Total	25 ul	x 16 =	400 ul

MasterEp F

FW [F]	2.5 ul	x 16 =	40 ul
RV [mKate]	2.5 ul	x 16 =	40 ul
2 x MasterMix TAQ	12.5 ul	x 16 =	200 ul
MilliQ	7.5 ul	x 16 =	120 ul
Total	25 ul	x 16 =	400 ul

Positive controls:

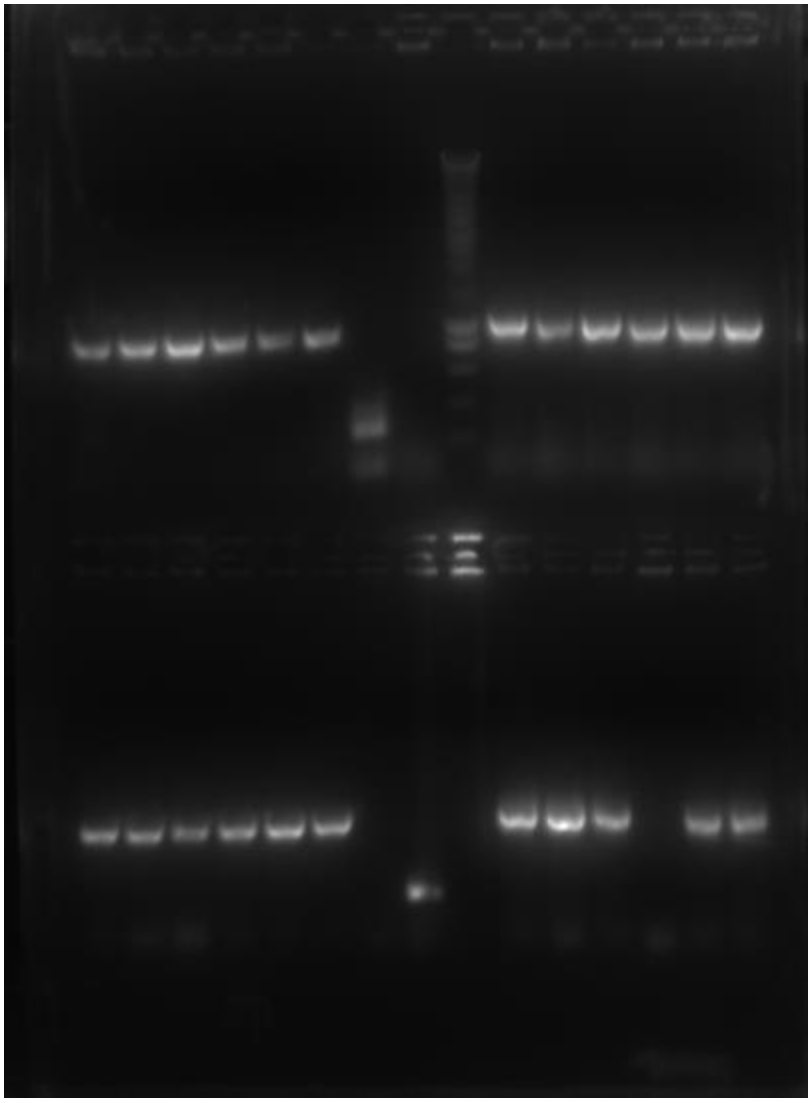
FW [F]	2.5 ul
RV [F] good	2.5 ul
2 x MasterMix TAQ	12.5 ul
MilliQ	7.5 ul
Total	25 ul

FW [J]	2.5 ul
RV [J]	2.5 ul
2 x MasterMix TAQ	12.5 ul

MilliQ	7.5 ul
Total	25 ul

Innoculated all colonies (1 to 6) from each culture (1 and 2). For promoter J culture 1 was used to take a positive (picked colony 3) and a negative (colony 7) control. For promoter F culture 2 was used to take a positive (picked colony 1) and a negative (colony 7) control.

PCR result
Loaded 10 ul of the 30 ul sample plus loading dye on 1 % agarose at 100 V for …. minutes.



11.08.14
Joan
Send the LD2 & LD3 constructs for sequencing

12.08.14
Joan
Miniprep LD2 culture 1&2; LD3 culture 1; and B0017.

13.08.14
Joan
Miniprep LD3 culture 2.

Grow on shakeflasks 4 single colonies of the DH5a cells carrying the plasmid with the synthesised N-genes.

14.08.14
Joan
Glycerol stock and miniprep the 4 single colonies grown overnight in the shakeflasks. The plasmid concentration after miniprepping the plasmids was:

- colony 1: 344 ng/ul
- colony 2: 301 ng/ul
- colony 3: 333 ng/ul
- colony 4: 317 ng/ul

Anne
Colony PCR of LD6 (p[F]-mkate-p[J]-mkate) transformants

Both transformants of culture 1 and culture 2 were observed. Therefore 2x 6 colonies were checked.

PCR MM 14x

FW [J]	2 ul [100uM]
RV makte	2 ul [100uM]
2 x MasterMix TAQ	175 ul
MilliQ	171 ul
Total	350ul

Colonies were ented with a pipet tip, inoculated in 25 ul MM, and streaked on LB+ CAM.

Cycle conditions

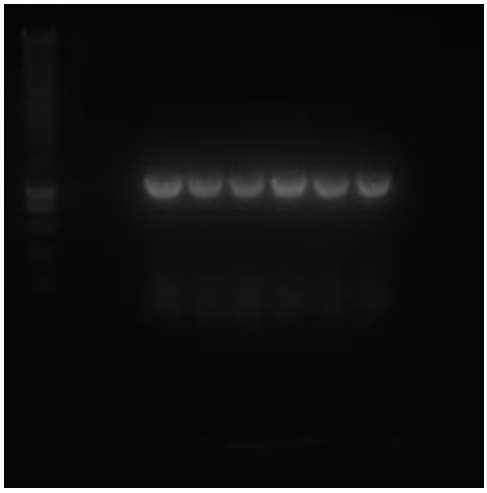
cycling conditions	minutes	°C	
1	5	94	
2	1	94	
3	1	56	
4	1	72	go to step 2, 29x
5	5	72	

→ 4 degrees
Negative control: GG CC50 bacterial colony

Run samples on 1% agerose gel, 100V 35 min

From left to right:

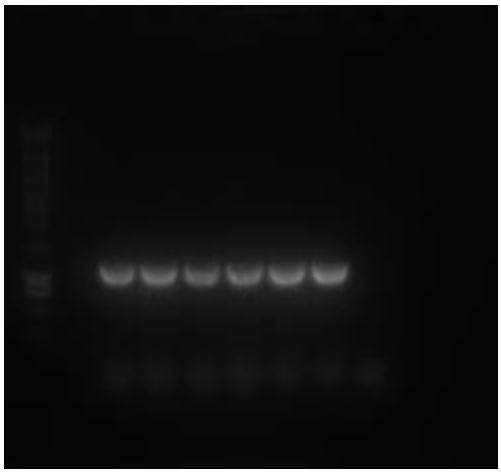
- smartladder
- open lane
- open lane
- sample 1-6 c1 p[F]-mkate-p[J]-mkate (approx. 1200 bp)



Expected fragments were observed

From left to right:

- smartladder
- open lane
- sample 1-6 c2 p[F]-mkate-p[J]-mkate (approx. 1200 bp)
- negative control

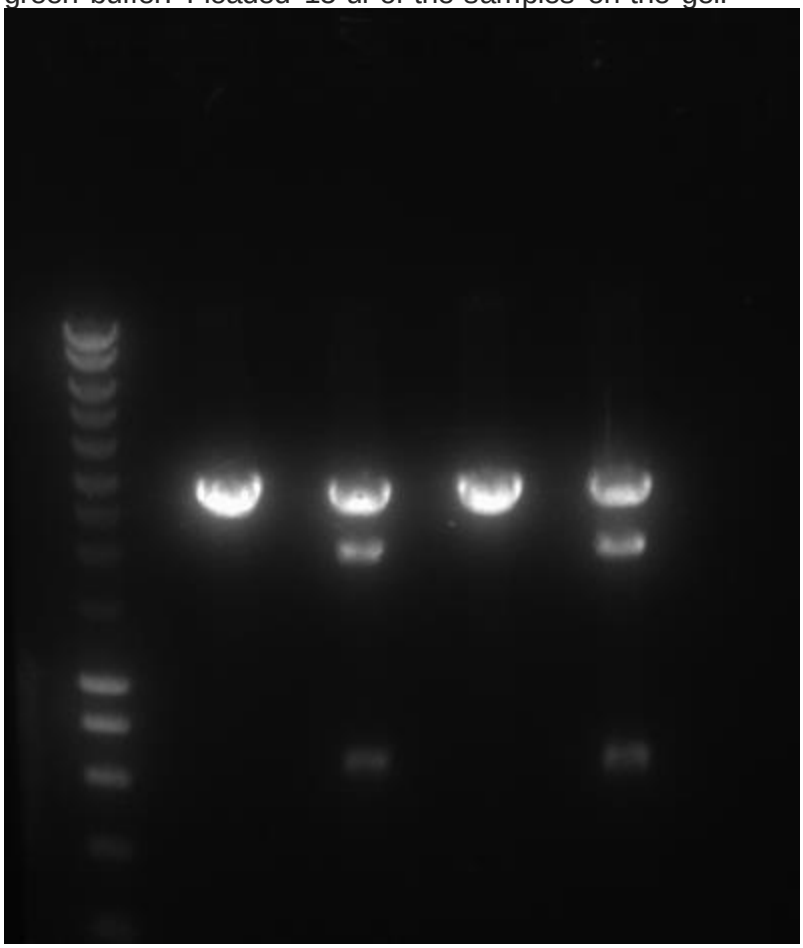


expected fragments were observed

15.08.2014
Janna
Restriction synthesized p[Rham]-N-enzymes
Did a restriction on the synthesized p[Rham]-N-enzymes construct with XbaI and ScaI, these are Thermo-Scientific restriction enzymes. I made two restrictions for two cultures of the sample.

	culture 1	344 ng/ul	culture 2	301 ng/ul
enzymes	XbaI	XbaI and ScaI	XbaI	ScaI
DNA	3.5 ul	3.5 ul	3.5 ul	3.5 ul
XbaI	1 ul	1 ul	1 ul	1 ul
ScaI	-	1 ul	-	1 ul
Buffer (green 10x)	2 ul	2 ul	2 ul	2 ul
milliQ	13.5 ul	12.5 ul	13.5 ul	12.5 ul
total	20 ul	20 ul	20 ul	20 ul

I incubated for 10 minutes at 37 C and after that loaded the samples on a 1 % agarose gel for 45 minutes at 100 V. I didn't add loading dye as this was already present in the green buffer. I loaded 15 ul of the samples on the gel.



Gel:

1. SmartLadder
2. N-enzymes culture 1 cut with XbaI
3. N-enzymes culture 1 cut with XbaI and ScaI
4. N-enzymes culture 2 cut with XbaI
5. N-enzymes culture 2 cut with XbaI and ScaI

Expected sizes of products:
A. pRham + N-enzymes approximately 2850 bp
B. whole backbone approximately 2800 bp
C. 1st part backbone cut with ScaI approximately 2000 bp
D. 2nd part backbone cut with ScaI approximately 800 bp

We can see on the gel that the samples cut only with XbaI have a band at size 2500-3000 bp. This could be number 1 only or number 2 only or number 1 and 2 together. If we now look at the samples cut with both XbaI and ScaI, we can see that there are three bands, which correlate with A, C and D. B could also be present, but the band at 2500-3000 bp is still very thick so product A is definitely present.

Joan
Inoculate cells containing the construct LD6. They are obtained from the strakes of the single colonies 1 and 8 that showed a positive band in the gel from the previous day.
At the end of the day, the grown cells were used to make glycerol stocks and minipreps.

Colony PCR of LD4 and LD5
19 colonies of the supposed LD4 and LD5 (p[F]+mKate2+N-genes; and p[J]+mKate2+N-genes) were used for colony PCR. The primers used were in all the cases:

1. FW mKate2
2. Standard iGEM reverse primer (VR)

The negative controls were LD2 (control for LD4) and LD3 (control for LD5), and they were amplified using the same pair of primers FW mKate2 and VR.

PCR mix:

FW mKate2	1 ul [20uM]
standard iGEM VR	1 ul [20uM]
2 x MasterMix TAQ	12.5 ul
MilliQ	10.5 ul
Total	25ul

Colonies were entered with a pipet tip.

Cycle conditions

cycling conditions	minutes	°C	
1	5	94	
2	1	94	
3	1	55 or 61*	
4	1	72	go to step 2, 29x
5	5	72	

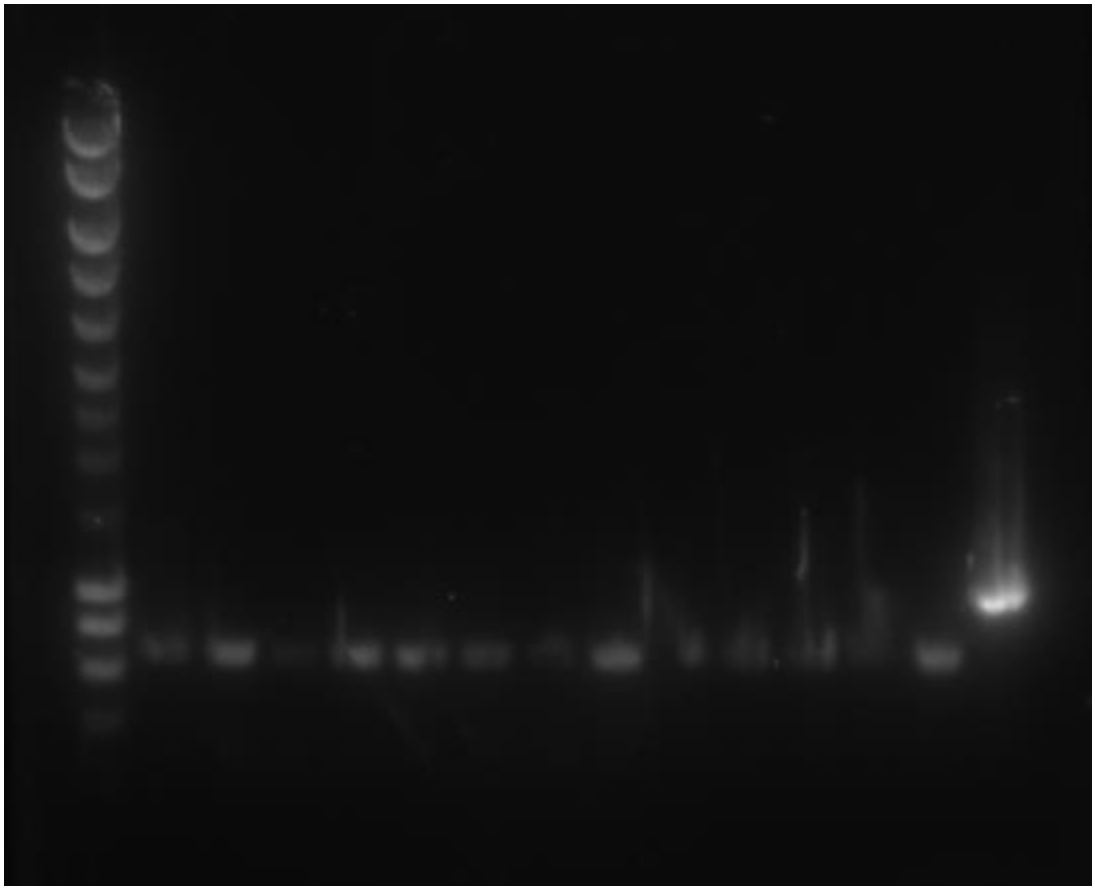
→ 4 degrees

*Half of the samples were PCRed with an annealing temperature of 55°C, and the other half at 61°C. That decision was made in base of the big difference in the melting temperature (™) of the forward and reverse primers (69 and 60°C respectively).

Run samples on 1% agarose gel, 100V 35 min

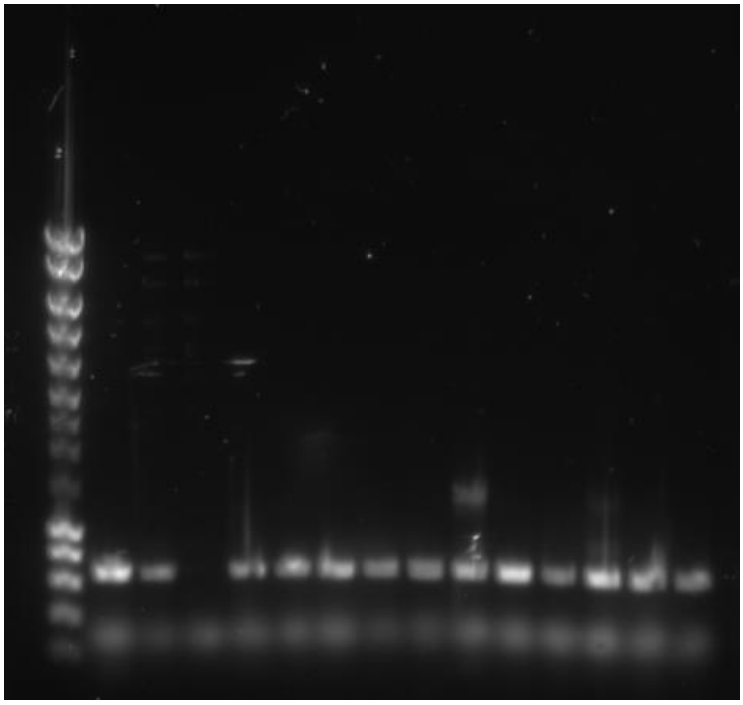
From left to right:

- Lane 1. Smart ladder
- Lanes 2-11. Colonies 2-11 LD4
- Lanes 12-14. Colonies 13-15 LD4
- Lane 15. Negative control LD4



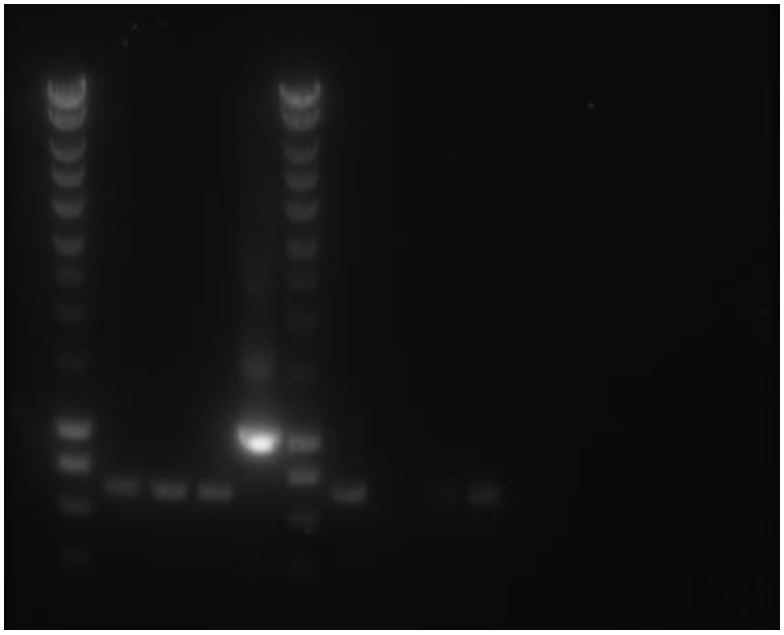
From left to right:

- Lane 1. Smart ladder
- Lane 2. Colony 1 LD5
- Lane 3. Colony 3 LD5
- Lanes 4-15. Colonies 5-16 LD5



From left to right:

- Lane 1. Smart ladder
- Lanes 2-4. Colonies 17-19 LD5
- Lane 5. Negative control LD5
- Lane 6. Smart ladder
- Lanes 7-10. Colonies 16-19 LD4



18.08.14
Joan
Colony PCR LD4 and LD5: 6 colonies of each construct and a negative control of each.

PCR mix:

FW mKate2	2.5 ul [5uM]
RV p[Rhamnose]	2.5 ul [5uM]
2 x MasterMix TAQ	12.5 ul
MilliQ	7.5 ul
Total	25ul

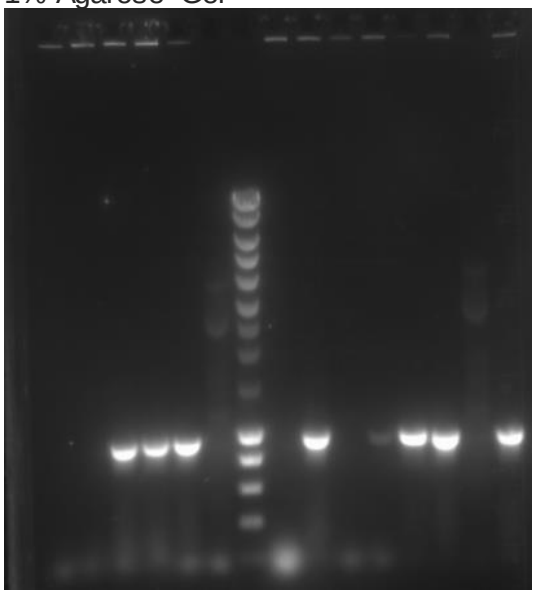
Colonies were entered with a pipet tip.

Cycle conditions

cycling conditions	minutes	°C	
1	5	94	
2	1	94	
3	1	55 or 59*	
4	1	72	go to step 2, 29x
5	5	72	

→ 4 degrees
*Colonies from 1-3 of each culture were PCRed with an Annealing temperature (Ta) of 55°C whereas colonies 4-6 at a Ta of 59°C

1% Agarose Gel



From left to right:
LD4 col 1-5; LD4's negative control; smart ladder; LD5 col 1-6; LD5s negative control; LD4 colony 6.

Transformation
Transform the land mine constructs LD1, 2, 3 and 6 on the working strain (BL21(DE3)).

Plate the transformed cells: 100uL and concentrate of each transformant on plates with LB+Chloramfenicol.

Grow on Shake-flasks
Start growing on shake-flasks LD4 colonies 3 and 5; and LD5 colonies 2 and 5.

19.08.14
Joan
Glycerol stock + Miniprep LD4 colonies 3 and 5; and LD5 colonies 2 and 5.

Start preculture of LD4 colony 6 and LD5 colony 6 on shake-flasks.

Colony PCR the transformed LD 1, 2, 3 and 6 on BL21 (3 colonies of each). The primers used were:

Construct	Forward primer	Reverse primer
LD1	mkate FW	mkate RV
LD2	pF FW	mkate RV
LD3	pJ FW	mkate RV
LD6	pF FW	pJ RV

PCR mix:

FW primer	2.5 ul [5uM]
RV primer	2.5 ul [5uM]
2 x MasterMix TAQ	12.5 ul
MilliQ	7.5 ul
Total	25ul

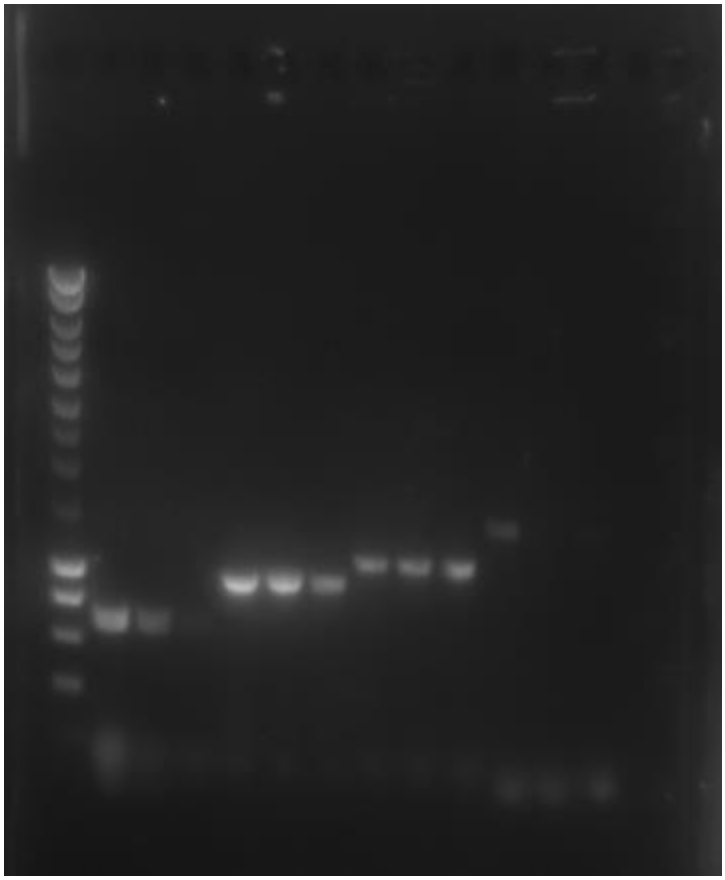
Colonies were entered with a pipet tip.
Cycle conditions

cycling conditions	Time	°C	
1	5'	94	

2	1’	94	
3	1’ 20”	62	
4	1’	72	go to step 2, 29x
5	5’	72	

→ 4 degrees

Agarose gel



From left to right: Smart Ladder; LD1 on BL21 col 1-3 (expected size 750bp); LD2 on BL21 col 1-3 (expected size 1000bp); LD3 on BL21 col 1-3 (expected size 1200bp); LD6 on BL21 col 1-3 (expected size 1500bp);

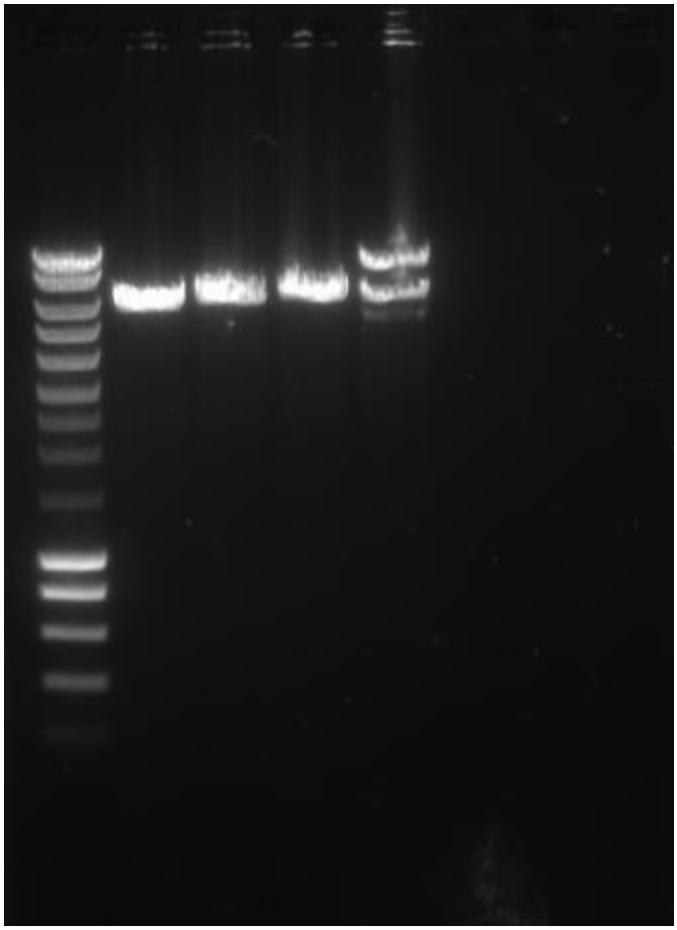
Check if the LD4 and LD5 constructs have illegal EcoRI sites
To check for illegal EcoRI sites, the miniprepped LD4 and LD5 plasmids (colonies 3, 5 and 2, 5 respectively) were digested with EcoRI-HF. The desired plasmid should show a single band on a gel, whereas if an illegal EcoRI site is present inside the Biobrick there should be 2 bands (remember that the Biobrick prefix has already an EcoRI site).

Digestion mix

	ul
DNA (ca. 300ng/uL)	6
EcorRI-hf	0.5
cutsmart	3
MQ	20.5
Total	30

Digestion time: 1 hour
Digestion temperature: 37°C

Agarose gel
From left to right Smart Ladder; LD4 col3 and 5; LD5 col 2 and 5. Observing the results, we see that LD5 colony 5 has an illegal EcoRI site, whereas the rest do not. For the rest (LD4 col3 and 5; LD5 col 2), sequencing will give a definite idea if they are the desired constructs.



20.08.14
Joan
Sequencing
Sequencing LD4 col3 and 5; LD5 col 2

Transformation
Transform LD4 col3 and 5; LD5 col 2 into the working strain BL21(DE3).

21.08.14
Joan
Colony PCR of the transformed LD4 and LD5 in BL21 to check if they were properly transformed. 3 colonies of each construct (2 from LD4 and 1 from LD5) were used, and a negative control for each as well.
LD2 was used as a negative control for LD4.
LD3 was used as a negative control for LD5.

PCR mix:

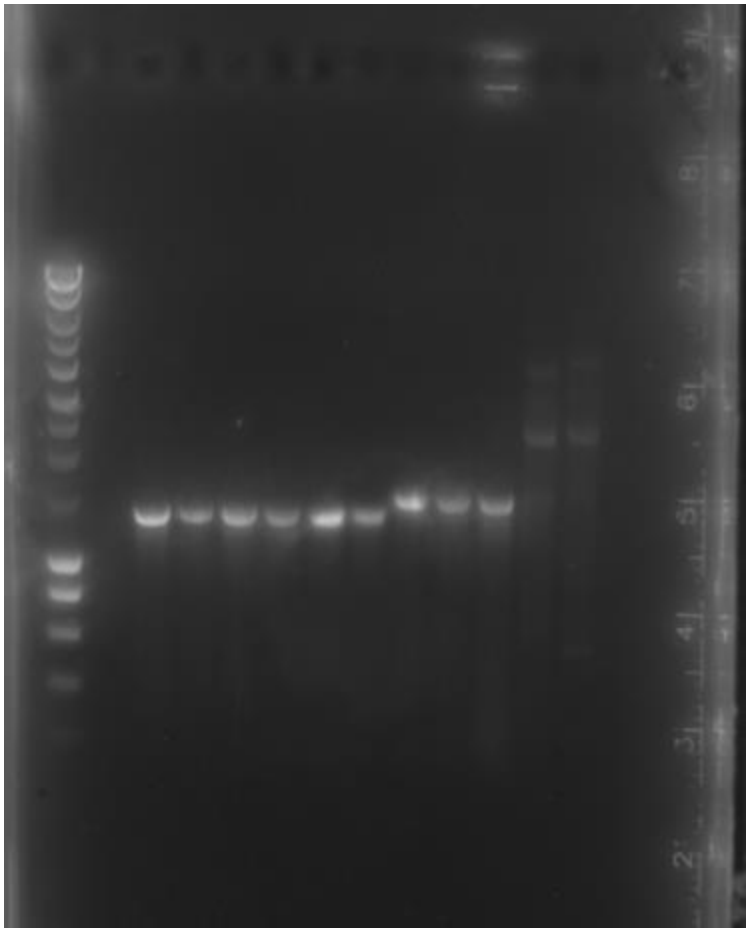
VF2 standard FW primer	0.5 ul [20uM]
p[Rhamnose] RV primer	2.5 ul [5uM]
2 x MasterMix TAQ	12.5 ul
MilliQ	9.5 ul
Total	25ul

Colonies were entered with a pipet tip.
Cycle conditions

cycling conditions	Time	°C	
1	5’	94	
2	1’	94	
3	1’	55	
4	1’ 30’’	72	go to step 2, 29x
5	5’	72	

→ 4 degrees

1% Agarose gel
From left to right: Smart ladder; LD4 3 colonies coming from the transformed LD4 colony 3; LD4 3 colonies coming from the transformed LD4 colony 5; LD5 3 colonies coming from the transformed LD5 colony 2; LD4 negative’s control; LD5’s negative control.
Columns 2-7 have an expected size of approximately 1250bp.
Columns 8-10 have an expected size of approximately 1600bp.



22.08.14
Joan
Glycerol Stock the working strains (on BL21(DE3)).

25.08.14
Joan

LD plate reader assay
Analyse the LD constructs (1-6) on the plate reader.
The Land mine promoters were not induced: the cells were mixed with LB alone instead of with the chemicals to be tested (2,4-DNT, 2,4,6-TNT and 1,3-DNB). The purpose of the experiment was to see if LB itself already induces these promoters, which is not desired, as the promoters are desired to be only dependent on land mine compounds
As a negative control, LB alone (without cells) was also used in the plate reader.
Different OD values were used for each LD construct: 2, 1, 0.5, 0.25 and 0.125.
The samples distribution on the plate reader was*:

A12: LD6, OD 100%	B12: LD6, OD 50%	C12: LD6, OD 25%	D12: LD6, OD 12.5%	E12: LD6, OD 6.25%	
A11: LD6, OD 100%	B11: LD6, OD 50%	C11: LD6, OD 25%	D11: LD6, OD 12.5%	E11: LD6, OD 6.25%	
A10: LD5	LD5	LD5	LD5	LD5	

A9: LD5	LD5	LD5	LD5	LD5	
A8: LD4	LD4	LD4	LD4	LD4	
A7: LD4	LD4	LD4	LD4	LD4	
A6: LD3	LD3	LD3	LD3	LD3	
A5: LD3	LD3	LD3	LD3	E5: LD3	
A4: LD2, OD 100%	B4: LD2, OD 50%	C4: LD2, OD 25%	D4: LD2, OD 12.5%	E4: LD2, OD 6.25%	
A3: LD2, OD 100%	B3: LD2, OD 50%	C3: LD2, OD 25%	D3: LD2, OD 12.5%	E3: LD2, OD 6.25%	
A2: LD1, OD 100%	B1: LD1, OD 50%	C1: LD1, OD 25%	D1: LD1, OD 12.5%	E1: LD1, OD 6.25%	F2: LB alone
A1: LD1, OD 100%	B1: LD1, OD 50%	C1: LD1, OD 25%	D1: LD1, OD 12.5%	E1: LD1, OD 6.25%	F1: LB alone

* From A10 to E5 they follow the same pattern as the rest

28.08.2014
Janna
Transformation of mKate in BL21
Made the following transformation:
1 ul mKate plasmid (plasmid 43 with a concentration of 94 ng/ul) added to 30 ul of BL21(DE3) competent cells
Followed the transformation in home-made competent cells protocol and made three plates with each 100 ul:
Ampicillin - some growth (5 cfu)
Chloramphenicol - no growth
No antibiotics - lots of growth
The transformation worked, but later on the colonies on the ampicillin plate were nowhere to be found, so new ones were made anyway.

12.09.2014
Joan
Aim: generate a positive control for the Land Mine experiments. The original mKate2 plasmid has the fluorescent protein regulated by a temperature inducible promoter (pCMV IE), and it has a KAN_r/Neomycin_r marker.

What was done: Transform the original mkate2 plasmid in BL21.
The transformants were plated on both Kan and Amp plates. The negative control was plated on Ampicillin, Kanamycin and on LB alone.

15.09.2014
Joan
Check miscibility of Tris buffer and Acetonitrile at different ratios (1:2, 1:1, 2:1).

Check for the transformants: PCR mkate2 from the transformed original mKate2 plasmid. 3 colonies from both the Kanamycin and Ampicillin plates, as well as one colony from the plated negative control on Kanamycin were used.

PCR mix:

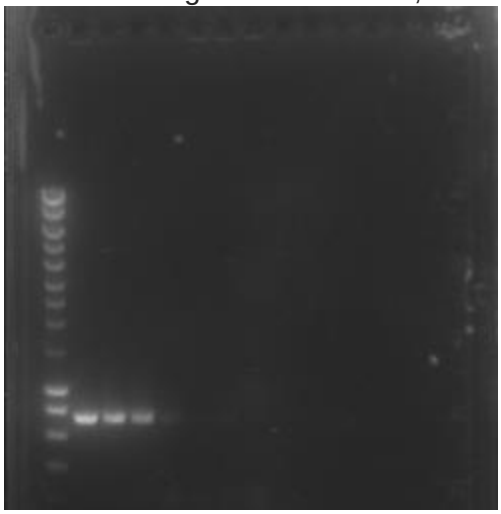
mKate2 FW primer	2.5 ul [5uM]
mKate2 RV primer	2.5 ul [5uM]
2 x MasterMix TAQ	12.5 ul
MilliQ	7.5 ul
Total	25ul

Colonies were entered with a pipet tip.
Cycle conditions

cycling conditions	Time	°C	
1	5’	94	
2	1’	94	
3	1’	62	
4	1’	72	go to step 2, 29x
5	5’	72	

→ 4 degrees

1% Agarose gel
From left to right: Smart ladder; 3 Amp colonies; 3 Kanamycin colonies; Negative control



16.09.2014
Joan
Objective: generate the positive control for the land mine module (LD0) which consists of mKate2 regulated by a constitutive promoter (J23110 Anderson promoter)

Restrict the purified mKate2 from the electrophoresis gel of the day before using XbaI and PstI.

Digestion mix

	ul
DNA (ca. 25ng/uL)	20
XbaII-HF	0.5
PstI-HF	0.5
cutsmart	3
MQ	6
Total	30

Digestion time: 1 hour
Digestion temperature: 37°C

Ligate the restricted mKate2 into the restricted pSB1A2 plasmid containing the J23110 constitutive Anderson promoter

Ligation mix

	ul
mKate2 (cut X,P) [7ng/uL]	14.29
pSB1A2 backbone (cut S,P) [27.1 ng/uL]	1.75
Ligation buffer	2
T4 Ligase	2
MQ	0
Total	20.04

Insert size: 700bp
Backbone size: 2070bp
Molar ratio (Insert:Backbone) of 3:1

Ligation time: 2 hours @ room temperature

17.09.2014
Joan

Transformation of LD0 into BL21(DE3) cells.

Inoculate on plates the transformed cells. The LD0 construct should grow on Ampicillin plates.

18.09.2014
Joan

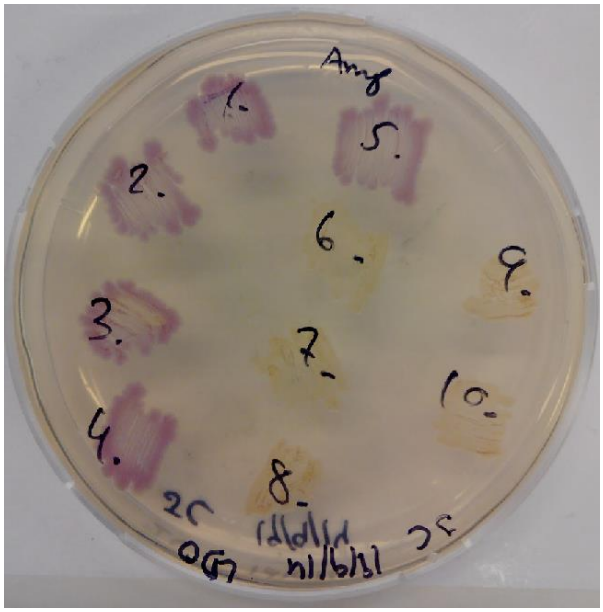
LD plate reader assay
Compound: 1,3-Dinitrobenzene (DNB)
Samples tested:

+30 mg/L DNB	LD1	LD2	LD3	LD4 non induced	LD5 non induced	LD6	LD4 induced (1% Rhamnose)	LD5 induced (1% Rhamnose)
0 mg/L DNB + Tris buffer + 30uL/L acetonitrile	LD1	LD2	LD3	LD4 non induced	LD5 non induced	LD6	LD0 (positive control)	-
0 mg/L DNB + Tris buffer	LD1	LD2	LD3	LD4 non induced	LD5 non induced	LD6	-	-

OD of the culture at the moment of mixing with the DNB solution: approximatelly 0.2
Final OD in the well: approximately 0.1

The final mixture contained 50% of cells (on LB) and 50% of chemical solution (DNB(+Tris buffer) and/or acetonitrile)).

19.09.2014
Joan
Strake colonies of LD0. They are visibly constitutively expressing mKate2:



22.09.2014
Joan

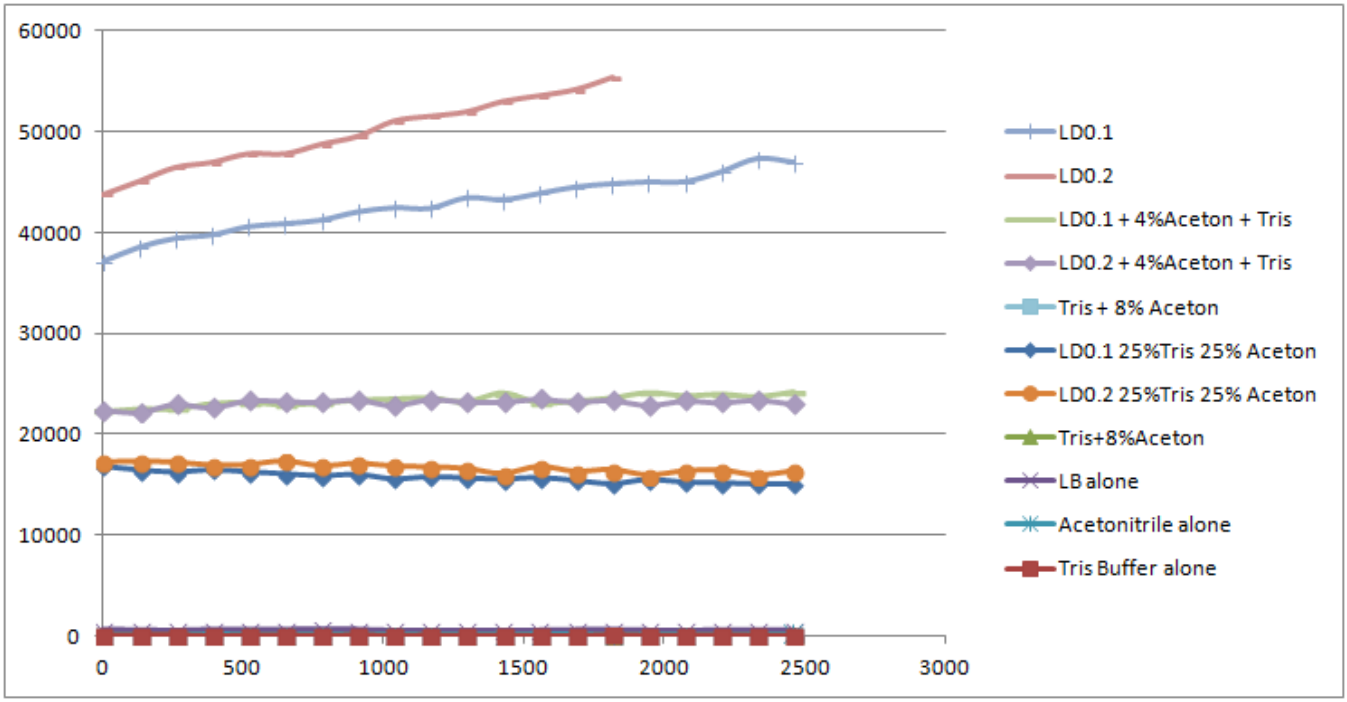
LD plate reader assay
To test: only the positive and negative controls of the LD module.
Two colonies of the positive control were tested (LD0.1 and LD0.2)
The negative controls (different medias: Tris buffer, acetonitrile and/ or LB) were tested for signal at the conditions tested.

Samples tested:

LD0.1	LD0.2	LD0.1 + 4%Acetonitrile + Tris buffer	LD0.2 + 4%Acetonitrile + Tris buffer	8%Acetonitrile + Tris buffer alone	
LD0.1 + 25%Acetonitrile + 25% Tris buffer	LD0.2 + 25%Acetonitrile + 25% Tris buffer	Tris buffer + 8% acetonitrile	LB alone	Acetonitrile alone	
0 mg/L DNB + Tris buffer	LD1	LD2	LD3	LD4 non induced	Tris buffer alone

OD of the culture at the moment of mixing with the DNB solution: approximatelly 0.3
Final OD in the well: approximately 0.15, except for the first two LD0.1 and LD0.2 samples, where the OD was 0.3.

The final mixture contained 50% of cells (on LB) and 50% of chemical solution (DNB(+Tris buffer) and/or acetonitrile)).



The results clearly show that whereas all the LD0 cultures (positive controls) showed fluorescence, the negative controls (different medias without cells: Tris buffer, acetonitrile and/ or LB) did not. The fact that LD0.1 and LD0.2 showed more fluorescence than the other LD0 samples is because the first two had double concentration than the rest.

23.09.2014
Joan

LD plate reader assay
Compound: 1,3-Dinitrobenzene (DNB)
Samples tested:

+300 mg/L DNB	LD0	LD1	LD2	LD3
+100 mg/L DNB	LD0	LD1	LD2	LD3
+33 mg/L DNB	LD0	LD1	LD2	LD3
0 mg/L DNB + Tris buffer	LD0	LD1	LD2	LD3
0 mg/L DNB + Tris buffer + 4% acetonitrile	LD0	LD1	LD2	LD3
0 mg/L DNB + LB	LD0	LD1	LD2	LD3

Results: No clear induction pattern is observed

PCR check of the cultures LD2, 4 and 6

PCR mix:

p[F] FW primer	2.5 ul [20uM]
mKate2 RV primer	2.5 ul [5uM]
2 x MasterMix TAQ	12.5 ul
MilliQ	7.5 ul
Total	25ul

The cells were obtained from a centrifuged pellet of the culture and entered the PCR tube with a pipet tip.

Cycle conditions

cycling conditions	Time	°C	
1	5’	94	
2	1’	94	
3	1’	60	
4	1’ 10”	72	go to step 2, 29x
5	5’	72	

→ 4 degrees

25.09.2014
Joan

LD plate reader assay
Compound: 2,4-Dinitrotoluene (DNT)

Samples tested: LD0, LD1, LD2, LD3, LD4, LD5 induced (1% Rhamnose), LD6, LD4 (col5) induced (1% Rhamnose), LD5 non-induced, LD2 (col2), LD4 (col5) non-induced

Conditions tested: 300, 200, 100, 50, 25 mg/L DNT, Tris buffer alone, Tris buffer+4%Acetonitrile
Results: No clear induction pattern is observed

29.09.2014
Joan

LD plate reader assay

Compound: 2,4-Dinitrotoluene (DNT)

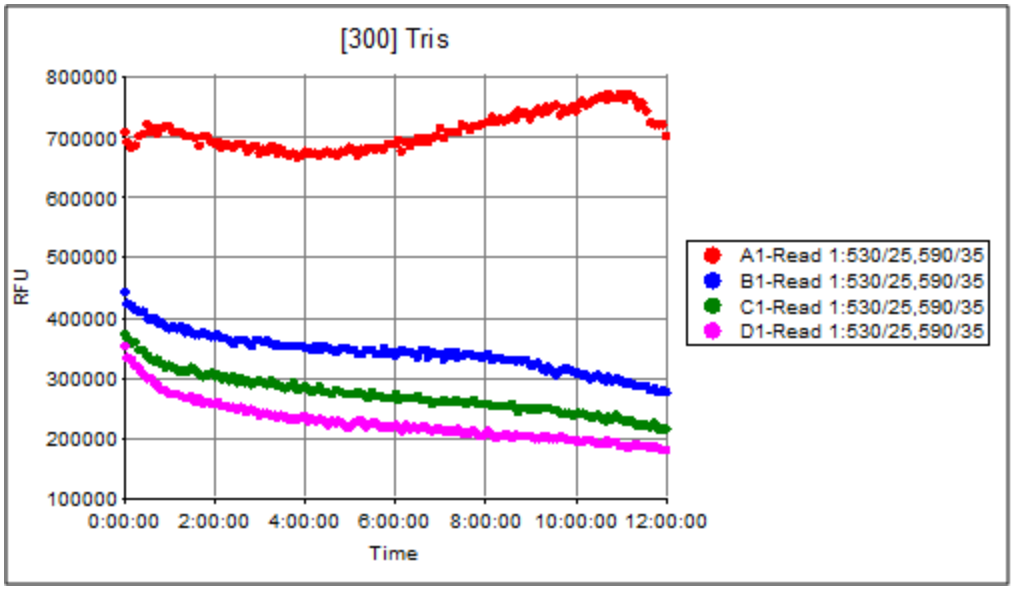
Samples tested (from A to D on the graphs): LD0, LD2, LD3, Negative control (BL21 without the plasmid)

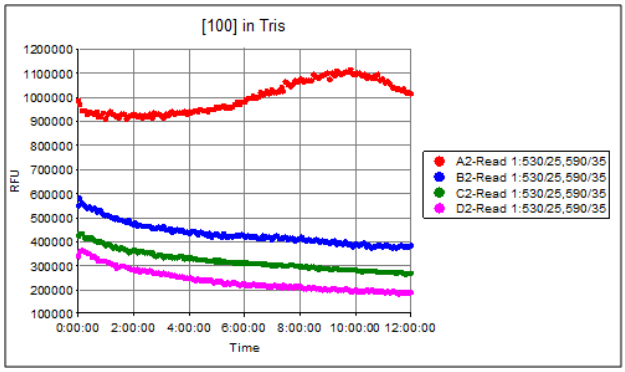
Conditions tested : 300, 100, 33, 0 mg/L DNT.
Solvent:

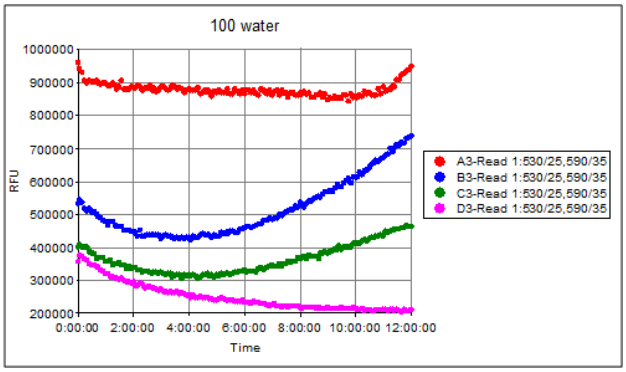
- For 300mg/L DNT the solvent was Tris buffer.
- For 100 and 33 mg/L DNT) the samples were prepared twice using either distilled water or Tris buffer as solvent.
- 0 mg/L DNT was either distilled water, or Tris buffer+8% acetonitrile.

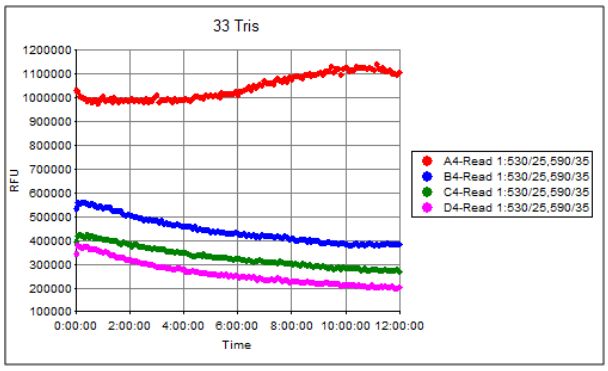
Therefore, 7 different conditions were tested (1 to 7 on the graphs)

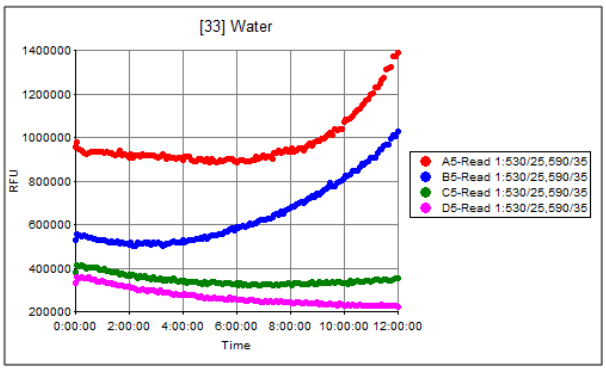
Results:
LD 2 and LD3 (letters B and C) present more fluorescence than the negative control (D)
Water seems to be the right solvent to do the test. Using Tris buffer no induction is observed.
When induced (and using distilled water as a solvent), the fluorescent signal of LD2 and LD3 (samples 3 and 5) increases much more than the non-induced sample (sample 7).

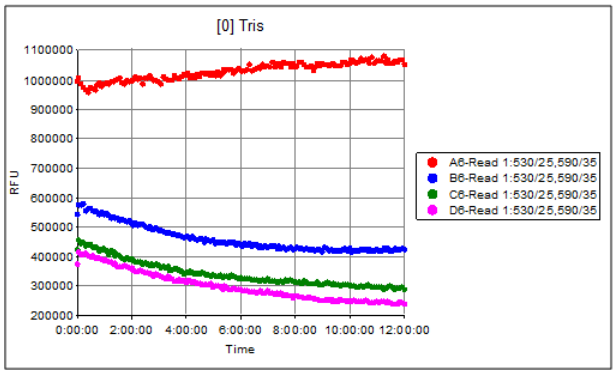


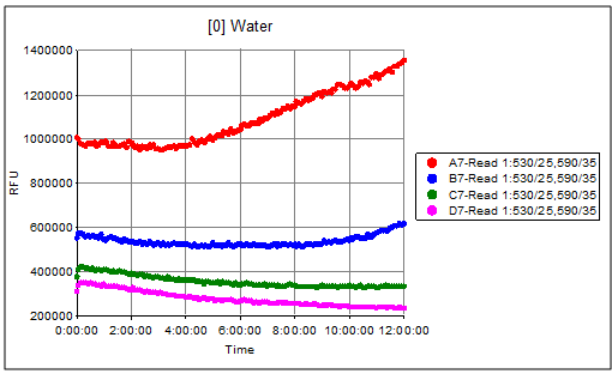






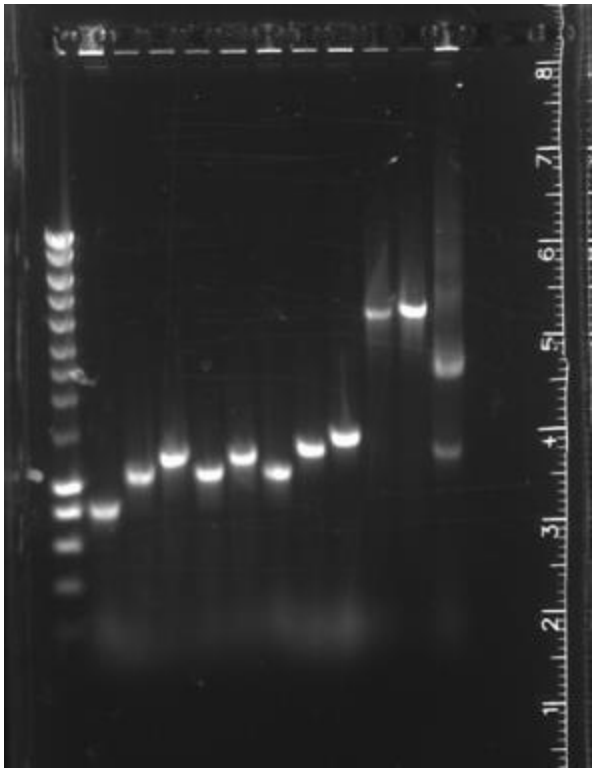






02.10.2014
Joan

Run a 1% agarose gel to verify the LD constructs



From left to right:

- Smart ladder
- LD 1, 2, 3, 4 and 5 amplified with standard forward (VF2) and mKate2 reverse primers
- LD 1, 2, 3, 4, 5 and 6 amplified with standard forward (VF2) and standard reverse (VR) primers

Week 40
Anne
Check glycerol stock with LD constructs by colony PCR
Streak stocks to single colonies on LB + CAM, incubate o/n at 37 degrees.
Take single colony as template for PCR

Samples to PCR:
LD1 t/m LD 5 were checked with mkate reverse primer and standard fw primer.
LD1 t/m LD6 were checked with standard reverse and standard fw primers.
PCR MM mkate reverse primer and standard fw primer.

		ul
8X	MM	100
	FW 100uM	1
	RV 100uM	1
	MQ	98

PCR MM with standard reverse and standard fw primers.

		ul
8X	MM	100
	FW 100uM	1
	RV 5uM	20
	MQ	79

Take 25 ul of the MM per reaction. Add some bacteria of a single colony to the mix.
PCR conditions for LD1 /tm LD5 withmkate reverse primer and standard fw primer and LD 1 t/m LD 3 with standard reverse and standard fw primers:

cycling conditions	minutes	°C	
1	5	94	
2	1	94	
3	1	55	
4	1.5	72	go to step 2, 29x
5	5	72	

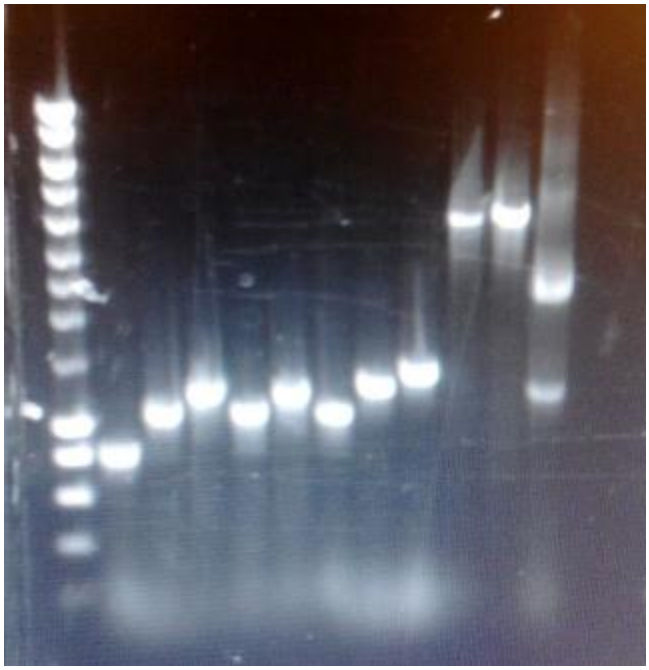
PCR conditions for LD 4 t/m LD 6 with standard reverse and standard fw primers:

cycling conditions	minutes	°C	
1	5	94	
2	1	94	
3	1	55	
4	4.5	72	go to step 2, 29x
5	5	72	

Add 5 ul loading dye to the samples and run them on a 1% agerose gell, 100V, 40 min.
From left to right:
smartladder marker

- LD1 mkate RV, standard FW, 900 bp
- LD2 mkate RV, standard FW, 1250 bp
- LD3 mkate RV, standard FW, 1350 bp
- LD4 mkate RV, standard FW, 1250 bp
- LD5 mkate RV, standard FW, 1350 bp
- LD1 standard RV, standard FW, 1000 bp
- LD2 standard RV, standard FW, 1350 bp

- LD3 standard RV, standard FW, 1450 bp
- LD4 standard RV, standard FW, 4350 bp
- LD5 standard RV, standard FW, 4450 bp
- LD6 standard RV, standard FW, 2200 bp



All PCR samples give back the expected values, except for sample LD6. Besides the expected size, there's a smaller fragment observed as well. This construct needs to be rechecked.